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**METHOD OF PRESERVING HUMAN COPPER AND ZINC TYPE  
SUPEROXIDE DISMUTASE**

[Hito Cu, Zn Gata Sūpāokishido Jisumutāze no Antei Hozonpō]

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1. Title: METHOD OF PRESERVING HUMAN COPPER AND ZINC TYPE  
SUPEROXIDE DISMUTASE

2. Claim

1. A method of preserving human copper and zinc type superoxide dismutase characterized in that said superoxide dismutase is mixed with at least one sugar selected from the group consisting of disaccharides, ketose monosaccharides, and sugar alcohols.

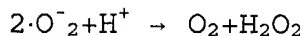
3. Detailed Description of the Invention

**Industrial Field of Application**

The present invention relates to a method employing a specified sugar to preserve human Cu and Zn type superoxide dismutase (abbreviated hereinbelow to "SOD") in a frozen state.

**Prior Art**

Human SOD is an enzyme which eliminates superoxide in the dismutation reaction shown below.



Thus, human SOD has attracted attention as a therapeutic drug effective for tissue damage (eg, inflammation, degenerative arthritis, chronic rheumatoid arthritis, damage from radiation exposure, damage from ultraviolet exposure, oxygen omental

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<sup>1</sup> Numbers in the margin indicate pagination in the foreign text.

disease in immature infants, cataracts, side effects of antineoplastics such as Adriamycin, damage accompanying restoration of circulation to areas deprived of blood, and the like) in the body caused by superoxide.

No decrease in the enzymatic action of human SOD is observed when this protein is subjected to freezing and thawing or freeze-drying processes, nor is formation of insoluble matter visible to the naked eye. However, human SOD subjected to analysis by sodium dodecyl sulfate — polyacrylamide electrophoresis, high-performance gel filtration liquid chromatography, and the like produces by-products consisting mostly of dimers.

Thus, the use of human SOD in pharmaceutical products necessitates its stable storage. However, by-products resulting

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from the storage process may have allergenic side effects; the generation of such substances must be prevented.

Most proteins are known to generate by-products, undergo denaturation rendering them insoluble and the like, and lose their biological activity when subjected to freezing and thawing or freeze-drying processes. Such denaturation is known to be preventable by addition to the protein solution of biological polymeric compounds such as albumin, DNA, carrageenan, dextran, or starch; amino acids; polyethylene glycol; or glycerol prior to freezing and thawing or freeze-drying.

However, the use of animal-derived albumin, DNA,

carrageenan, starch and the like is undesirable in pharmaceutical products containing human SOD since such substances may be allergenic. The use of human-derived substances, presenting sourcing difficulties and highly expensive, is not practical.

Conventional methods of preserving SOD include a method (US Patent No. 3,637, 64<sup>2</sup>) of preserving bovine SOD (tradename: Orgothin<sup>3</sup>, Diagnostic Data Co.). Denaturation of bovine SOD is 25 percent or greater with freeze-drying. However, by combining pentose and hexose (for example, galactose, fructose, fucose, arabinose, glucose, mannose, and sucrose) with bovine SOD prior to freeze-drying, denaturation of bovine SOD is prevented.

However, even with the combination of aldose monosaccharides such as galactose, arabinose, glucose, and the like to human SOD prior to freeze-drying, analysis by anion-exchange chromatography reveals denaturation (Comparative Example 3). Thus, the use of aldose monosaccharides to prevent denaturation of human SOD during freezing or freeze-drying is undesirable.

#### **Problems to Be Solved by the Invention**

The object of the present invention is to provide a method of preserving in a frozen or freeze-dried state a solution of human SOD processed with specified sugars.

#### **Methods of Solving the Problems**

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<sup>2</sup> Last digit missing in Japanese.-Tr.

<sup>3</sup> Transliteration of Orugochin in Japanese.-Tr.

As the result of extensive research conducted to solve the above-described problems, the present inventors discovered that by mixing human SOD with at least one sugar selected from the group consisting of disaccharides, ketose monosaccharides, and sugar alcohols, human SOD can be preserved in a frozen or freeze-dried state; the present invention was devised on this basis.

That is, the present invention relates to a method of preserving human SOD characterized in that human SOD is preserved by mixing it with at least one sugar selected from the group consisting of disaccharides, ketose monosaccharides, and sugar alcohols.

A quantity of sugar 0.05-10 times by weight, preferably 0.1-6 times by weight, the quantity of human SOD is employed in the present invention.

The present invention is described in greater detail below.

Human SOD suitable for use in the present invention can be extracted and refined from human cells, tissue, organs, or the like (eg, red blood cells, livers, or placentas); obtained by genetic recombination of microbes so that they produce SOD with the same amino acid sequence as human SOD; or the like.

Examples of sugars used in the present invention for the preservation of human SOD in a frozen or freeze-dried state are sugar alcohols (for example, sorbitol, mannitol, inositol, and ribitol), disaccharides (for example, sucrose, trehalose, maltose, lactose, isomaltose, cellobiose), and ketose

monosaccharides (for example, fructose, xylulose, ribulose, and sedoheptulose). These sugars can be used singly or in combination.

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There are no specific restrictions on the method used to mix human SOD with the above-listed sugars. Examples are:

- (a) Direct addition of sugar to an SOD solution (1-100 mg/mL) followed by intimate mixing;
- (b) Addition of a sugar solution to an SOD solution (1-100 mg/mL) followed by intimate mixing; and
- (c) Direct addition of an SOD solution to a sugar solution followed by intimate mixing.

This processing can be conducted at a temperature of 0-40°C, preferably 0-15°C. There is no specific limit to the processing time after mixing.

The solvent used with the SOD solution or sugar solution of the present invention is not specifically limited. For example, water, physiological saline, phosphate buffer solution, and other buffer solutions can be used.

A suitable quantity of the sugar-containing human SOD solution thus prepared is charged to a vial or other suitable container, frozen at a temperature of -80 to -15°C, and stored as is at -80 to -15°C, or dried under a vacuum (200-500 mTorr) and stored at -80 to -10°C. In this manner, human SOD can be preserved in a frozen or freeze-dried state.

## Embodiments

The present invention is described in detail below on the basis of reference examples and embodiments. However, these embodiments do not limit the scope of the present invention.

Testing of the embodiments by polyacrylamide electrophoresis, high-performance gel filtration liquid chromatography, and anion-exchange chromatography was conducted according to the methods indicated below:

### (1) Polyacrylamide electrophoresis

Analysis (referred to hereinbelow as "electrophoretic analysis") of by-products produced in the storage of frozen or freeze-dried human SOD processed by addition of a specified sugar was conducted based on the Remury method (*Nature*, 227, 680 (1970)).

Minislabs of gel (length: 60 mm, width: 100 mm, separation portion: 45 mm, holes: 10) having a 3 percent gel concentration and a 12.5 percent separation gel concentration were prepared, 20  $\mu$ g of denatured sample was placed into each hole, and the samples were subjected to electrophoresis with a constant current of 20 mA. The electrophoretic samples were then dyed with Komashī Brilliant Blue R-250. Human SOD was confirmed at 20 KD (kilodaltons) (monomer) and by-products were identified at 40 KD using electrophoretic molecular weight markers from Farmacia.

### (2) High-performance gel filtration liquid chromatography

Analysis by high-performance gel filtration liquid

chromatography (referred to hereinbelow as "gel filtration analysis") of by-products produced in the storage of frozen or freeze-dried human SOD processed by addition of a specified sugar was conducted in the manner indicated below.

A TSK-3000 SW column (Tōyō Sōren) and an eluant in the form of 50 mM sodium phosphate — 0.2 M sodium chloride solution (pH 7) were employed with a flow rate of 0.7 mL/min. Use of high-performance gel filtration chromatographic molecular weight markers from Oriental Enzyme Co. confirmed human SOD at 40 KD and by-products were identified at 79 KD.

### (3) Anion-exchange chromatography

Analysis by anion-exchange chromatography (referred to as DEAE analysis hereinbelow) of change occurring in the storage of frozen or freeze-dried human SOD processed by addition of a specified sugar was conducted on a TS-DEAE 5 PW column (Tōyō Sōren) in the manner indicated below.

Anion-exchange chromatography was conducted by the linear gradient method using a TS-DEAE 5 PW column and eluants in the form of an A solution (20 mM tris acetate (pH 8.5)) and a B solution (20 mM tris acetate — 0.5 M sodium acetate (pH 8.5)) (the B solution was brought from 0 percent to 15 percent over 74 min) at a flow rate of 0.8 mL/min.

#### **Embodiment 1**

A 74 mg quantity of sorbitol, a sugar alcohol, was added to 0.37 mL of human SOD solution (human SOD 100 mg/1 mL of distilled

water) and distilled water was added to make 1 mL. This solution

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was charged to a vial, frozen at -20°C, and thawed at room temperature. The stability of human SOD when frozen and stored with the addition of sugar was examined by above-described gel permeation analysis (2) and DEAE analysis (3).

Gel permeation analysis revealed by-products at 79 KD. The quantities produced were 0.007 percent after five cycles, and 0.008 percent after 10 cycles of repeated freezing and thawing.

DEAE analysis did not reveal any denaturation of human SOD.

#### **Embodiment 2**

The stability of human SOD when frozen and stored with the addition of sugar was examined in the same manner as in Embodiment 1 except for the substitution of inositol, a sugar alcohol, for sorbitol.

The quantity of by-product identified at 79 KD by gel permeation analysis was 0.014 percent after five cycles, and 0.011 percent after 10 cycles of repeated freezing and thawing.

DEAE analysis did not reveal any denaturation of human SOD.

#### **Embodiment 3**

The stability of human SOD when frozen and stored with the addition of sugar was examined in the same manner as in Embodiment 1 except for the substitution of sucrose, a disaccharide, for sorbitol.

The quantity of by-product identified at 79 KD by gel

permeation analysis was 0.004 after five cycles and 0.008 percent after 10 cycles of repeated freezing and thawing.

DEAE analysis did not reveal any denaturation of human SOD.

#### **Embodiment 4**

The stability of human SOD when frozen and stored with the addition of sugar was examined in the same manner as in Embodiment 1 except for the substitution of trehalose, a disaccharide, for sorbitol.

The quantity of by-product identified at 79 KD by gel permeation analysis was 0.008 percent after five cycles, and 0.007 percent after 10 cycles of repeated freezing and thawing.

DEAE analysis did not reveal any denaturation of human SOD.

#### **Embodiment 5**

The stability of human SOD when frozen and stored with the addition of sugar was examined in the same manner as in Embodiment 1 except for the substitution of maltose, a disaccharide, for sorbitol.

The quantity of by-product identified at 79 KD by gel permeation analysis was 0.004 percent after five cycles, and 0.007 percent after 10 cycles of repeated freezing and thawing.

DEAE analysis did not reveal any denaturation of human SOD.

#### **Comparative Example 1**

The stability of human SOD when frozen was examined in the same manner as in Embodiment 1 except for the omission of

sorbitol.

The quantity of by-product identified at 79 KD by gel permeation analysis was 0.019 percent after five cycles, and 0.028 percent after 10 cycles of repeated freezing and thawing.

DEAE analysis did not reveal any denaturation of human SOD.

Table 1 shows the results of the examination of human SOD storage stability when frozen and thawed with the addition of sugars in Embodiments 1-5, and without sugar, in Comparative Example 1.

**Table 1**

Embodiment	Sugar Additive	Cycles	Test Method (2) 79 KD (%)	Test Method (3) Denaturation
1	Sorbitol	5 10	0.007 0.008	None
2	Inositol	5 10	0.004 0.011	None
3	Sucrose	5 10	0.004 0.008	None
4	Trehalose	5 10	0.008 0.007	None
5	Maltose	5 10	0.004 0.007	None
Comparative Example 1 (No Sugar)		5 10	0.019 0.028	None

**Embodiment 6**

A 100 mg quantity of sorbitol, a sugar alcohol, was added to 0.5 mL of human SOD solution (human SOD 100 mg/distilled water 1 mL) and distilled water was added to make 1 mL. The solution was charged to a vial, frozen at -80°C, and dried over night under a

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vacuum (200-500 mTorr). The freeze-dried human SOD obtained was dissolved in distilled water and the stability of freeze-dried human SOD was examined.

Gel permeation analysis revealed a 0.08 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 1).

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Embodiment 7**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of mannitol, a sugar alcohol, for sorbitol.

Gel permeation analysis revealed a 0.23 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 2).

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Embodiment 8**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of inositol, a sugar alcohol, for sorbitol.

Gel permeation analysis revealed a 0.11 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 3).

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Embodiment 9**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of sucrose, a disaccharide, for sorbitol.

Gel permeation analysis revealed a 0.04 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 4).

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Embodiment 10**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of trehalose, a disaccharide, for sorbitol.

Gel permeation analysis revealed a 0.05 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 5).

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Embodiment 11**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of maltose, a disaccharide, for sorbitol.

Gel permeation analysis revealed a 0.01 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 6).

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Embodiment 12**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of lactose, a disaccharide, for sorbitol.

Gel permeation analysis revealed a 0 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 7).

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Embodiment 13**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of fructose, a ketose monosaccharide, for sorbitol.

Gel permeation analysis revealed a 0.01 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 8).

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Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Comparative Example 2**

The stability of freeze-dried human SOD was examined in the same manner as in Embodiment 6 except for the omission of sorbitol.

Gel permeation analysis revealed a 0.45 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 12)

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Comparative Example 3**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of arabinose, a monosaccharide, for

sorbitol.

Gel permeation analysis revealed a 0.03 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 9)

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Comparative Example 4**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of glucose, a monosaccharide, for sorbitol.

Gel permeation analysis revealed a 0.01 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD (Fig. 10)

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

#### **Comparative Example 5**

The stability of freeze-dried human SOD with the addition of sugar was examined in the same manner as in Embodiment 6 except for the substitution of galactose, a monosaccharide, for sorbitol.

Gel permeation analysis revealed a 0.06 percent quantity of by-product at 79 KD.

DEAE analysis did not reveal any denaturation of human SOD

(Fig. 11)

Electrophoretic analysis did not reveal any by-products having a molecular weight of 40 KD.

Table 2 shows the results of the examination of freeze-dried human SOD storage stability with the addition of sugars in Embodiments 6-13 and Comparative Examples 3-5, and without sugar, in Comparative Example 2.

**Table 2**

Embodiment	Test Method (1) 40 KD	Test Method (2) 79 KD (%)	Test Method (3) Denaturation
6	None	0.08	None
7	Present	0.23	None
8	None	0.11	None
9	None	0.04	None
10	None	0.05	None
11	None	0.01	None
12	None	0	None
13	None	0.01	None
Comparative Ex. 2	Present	0.45	None
Comparative Ex. 3	None	0.03	Present
Comparative Ex. 4	None	0.01	Present
Comparative Ex. 5	None	0.06	Present

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#### **Embodiment 14**

A 5 mg quantity of sorbitol, a sugar alcohol, was added to 0.5 mL of human SOD solution (human SOD 100 mg/distilled water 1 mL) and distilled water was added to make 1 mL. The solution was charged to a vial, frozen at -20°C, and dried over night under a vacuum (200-500 mTorr). The freeze-dried human SOD obtained was dissolved in distilled water and the stability of freeze-dried human SOD examined.

Gel permeation analysis revealed a 0.236 percent quantity of by-product at 79 KD.

#### **Embodiment 15**

The stability of freeze-dried human SOD with addition of sugar was examined in the same manner as in Embodiment 14 with the exception that 12.5 mg of sorbitol was employed.

Gel permeation analysis revealed a 0.104 percent quantity of by-product at 79 KD.

#### **Embodiment 16**

The stability of freeze-dried human SOD with addition of sugar was examined in the same manner as in Embodiment 14 with the exception that 25 mg of sorbitol was employed.

Gel permeation analysis revealed a 0.065 percent quantity of by-product at 79 KD.

#### **Embodiment 17**

The stability of freeze-dried human SOD with addition of sugar was examined in the same manner as in Embodiment 14 with

the exception that 50 mg of sorbitol was employed.

Gel permeation analysis revealed a 0.038 percent quantity of by-product at 79 KD.

#### **Embodiment 18**

The stability of freeze-dried human SOD with addition of sugar was examined in the same manner as in Embodiment 14 with the exception that 100 mg of sorbitol was employed.

Gel permeation analysis revealed a 0.012 percent quantity of by-product at 79 KD.

#### **Embodiment 19**

The stability of freeze-dried human SOD with addition of sugar was examined in the same manner as in Embodiment 14 with the exception that 200 mg of sorbitol was employed.

Gel permeation analysis revealed a 0.026 percent quantity of by-product at 79 KD.

#### **Embodiment 20**

The stability of freeze-dried human SOD with addition of sugar was examined in the same manner as in Embodiment 14 with the exception that 300 mg of sorbitol was employed.

Gel permeation analysis revealed 0 percent by-product at 79 KD.

#### **Comparative Example 6**

The stability of freeze-dried human SOD was examined in the same manner as in Embodiment 14 with the exception that sorbitol was omitted.

Gel permeation analysis revealed a 0.425 percent quantity of by-product at 79 KD.

Table 3 and Fig. 13 show the results of the examination of freeze-dried human SOD storage stability with the addition of sugars in Embodiments 14-20 and without sugar in Comparative Example 6.

**Table 3**

Embodiment	Human SOD (mg)	Sorbitol (mg)	Test Method (2) 74 KD (%)
14	50	5	0.236
15	50	12.5	0.104
16	50	25	0.065
17	50	50	0.038
18	50	100	0.012
19	50	200	0.026
20	50	300	0
Comparative Ex. 6	50	0	0.426

#### **Effect of the Invention**

Preservation of frozen or freeze-dried human SOD is possible by the method of the present invention (ie, a method by which human SOD is processed by mixing it with at least one sugar selected from the group consisting of disaccharides, ketose monosaccharides, and sugar alcohols to obtain a solution which is then frozen or freeze dried).

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#### **4. Brief Description of the Figures**

Fig. 1 shows the results of the DEAE analysis recorded in Embodiment 6.

Fig. 1 shows the results of the DEAE analysis recorded in Embodiment 6.

Fig. 2 shows the results of the DEAE analysis recorded in Embodiment 7.

Fig. 3 shows the results of the DEAE analysis recorded in Embodiment 8.

Fig. 4 shows the results of the DEAE analysis recorded in Embodiment 9.

Fig. 5 shows the results of the DEAE analysis recorded in Embodiment 10.

Fig. 6 shows the results of the DEAE analysis recorded in Embodiment 11.

Fig. 7 shows the results of the DEAE analysis recorded in Embodiment 12.

Fig. 8 shows the results of the DEAE analysis recorded in Embodiment 13.

Fig. 9 shows the results of the DEAE analysis recorded in Comparative Example 3.

Fig. 10 shows the results of the DEAE analysis recorded in Comparative Example 4.

Fig. 11 shows the results of the DEAE analysis recorded in Comparative Example 5.

Fig. 12 shows the results of the DEAE analysis recorded in

Comparative Example 2.

Fig. 13 shows the results of examination of the storage stability of freeze-dried human SOD with sugar addition in Embodiments 14-20 and without sugar in Comparative Example 6.

Fig. 1

Fig. 2

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Fig. 3

Fig. 4

Fig. 5

Fig. 6

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Fig. 7

Fig. 8

Fig. 9

Fig. 10

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Fig. 11

Fig. 12

Fig. 13

**CERTIFICATE OF CORRECTION (Form)**

September 27, 1988

Commissioner of Patents:

**1. Designation of Document**

Patent Application No. 63-135457

**2. Title of the Invention**

Method of Preserving Human Copper and Zinc Type Superoxide  
Dismutase

**3. Party Making Corrections**

**Relation to Document:** Applicant

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5. Number of Inventions Added in Corrections: None

6. Object of Correction:

Figures

7. Content of Corrections

(1) Figures 1-13 are hereby corrected as per accompanying sheets.

Fig. 1

Fig. 2

Fig. 3

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Fig. 4

Fig. 5

Fig. 6

Fig. 7

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Fig. 8

Fig. 9

Fig. 10

Fig. 11

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Fig. 12

Fig. 13

[#left] Amount Produced (%)

[#bottom] Sorbitol/Human SOD (W/W)